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REQUEST FOR FILING APPLICATION

Under Rule 53(a), (b) & (f)

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Use for Design or Utility Applications

RULE 53(f) NO DECLARATIONPATENT
APPLICATION

Hon. Commissioner of Patents

Atty. Dkt.

PM

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M#

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PAT/sw

Client Ref

Washington, DC 20231

Date:

November 20, 2000

Sir:

1. This is a Request for filing a new Patent Application (☐ Design ☒ Utility) entitled:2. (Complete) Title: **NOVEL NUCLEOTIDE SEQUENCES ENCODING THE PFKA GENE**

without a filing fee or Oath/Declaration but for which is enclosed the following:

3. ☒ Abstract 1 page(s).4. 32 Pages of Specification (only spec. and claims); 5. ☐ Specification in non-English language6. 19 Numbered claim(s); and7. ☒ Drawings: 2 sheet(s) ☐ 1 set informal; 8. ☒ formal of size: ☒ A4 ☐ 11"9. **DOMESTIC/INTERNATIONAL** priority is claimed under 35 USC 119(e)/120/365(c) based on the following provisional, nonprovisional and/or PCT international application(s):

Application No.	Filing Date	Application No.	Filing Date
(1)		(2)	
(3)		(4)	
(5)		(6)	

10. **FOREIGN** priority is claimed under 35 USC 119(a)-(d)/365(b) based on filing in GERMANY

Application No.	Filing Date	Application No.	Filing Date
(1) 199 56 133.8	23 November 1999	(2) 100 11 922.0	March 11, 2000
(3)		(4)	
(5)		<input type="checkbox"/> See 3 rd page for additional priorities	

11. 1 (No.) Certified copy (copies): ☒ attached; ☐ previously filed (date) _____
in U.S. Application No. _____ / _____ filed on _____12. ☐ This is a reissue of Patent No. _____13. ☐ See top first page re prior Provisional, National, International application(s) (X box only if info is there and do not complete corresponding item 14 or 15.)14. ☐ **Amend the specification** by inserting before the first line -- This is a ☐ Continuation-in-Part of:
☐ Divisional ☐ Continuation ☐ Substitute Application (MPEP 201.09)14(a) ☐ National Appln. No. _____ / _____ filed _____ (M# _____)14(b) ☐ International Appln. No. **PCT/** _____ filed _____ which _____
designated the U.S. --15. ☐ **Amend the specification** by inserting before the first line: --This application
claims the benefit of U.S. Provisional Application No. 60/ _____, filed _____ --16. Extension to date: ☐ concurrently filed ☒ not needed ☐ previously filed17. ☐ Small Entity Status is claimed (**pre-filing confirmation required**)17(a) ☐ Attached: _____ (No.) Small Entity Statement(s). (Since 9/8/00 Small Entity Statement not essential to make claim)17(b) ☐ See **NONPUBLICATION REQUEST** under Rule 213(a) attached (PAT-258)

18. ☐ Prior application is assigned to

by Assignment recorded _____ Reel _____ Frame _____

19. ☐ Attached:

20. This application is made by the following named inventor(s) (Double check instructions for accuracy.):
(Listing of inventor(s) not a requirement, but list if known)

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21. NOTE: FOR ADDITIONAL INVENTORS, check box ☐
and attach sheet with same information regarding additional inventors.

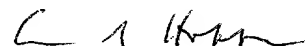
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APPLICATION UNDER UNITED STATES PATENT LAWS

Atty. Dkt. No. PM 274441
(M#)

Invention: NOVEL NUCLEOTIDE SEQUENCES ENCODING THE PFKA GENE

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This is a:

- ☐ Provisional Application
- ☒ Regular Utility Application
- ☐ Continuing Application
 - ☒ The contents of the parent are incorporated by reference
- ☐ PCT National Phase Application
- ☐ Design Application
- ☐ Reissue Application
- ☐ Plant Application
- ☐ Substitute Specification
Sub. Spec Filed _____
in App. No. _____ / _____
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Sub. Spec. filed _____
In App. No. _____ / _____

SPECIFICATION

Novel nucleotide sequences encoding the pfkA gene

This application claims priority from German Application Nos. 199 56 133.8, filed on November 23, 1999, and 100 11 922.0 filed March 11, 2000, the subject matter of each of which is hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention provides nucleotide sequences coding for the pfkA gene and a process for the fermentative production of L-amino acids, in particular L-lysine, using coryneform bacteria in which the pfkA gene is amplified.

2. Background Information

Amino acids, in particular L-lysine, are used in human medicine and in the pharmaceuticals industry, but in particular in animal nutrition.

It is known that amino acids are produced by fermentation of strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Due to their great significance, efforts are constantly being made to improve the production process. Improvements to the process may relate to measures concerning fermentation technology, for example stirring and oxygen supply, or to the composition of the nutrient media, such as for example sugar concentration during fermentation, or to working up of the product by, for example, ion exchange chromatography, or to the intrinsic performance characteristics of the microorganism itself.

The performance characteristics of these microorganisms are improved using methods of mutagenesis, selection and mutant selection. In this manner, strains are obtained which are resistant to antimetabolites, such as for example the lysine analogue S-(2-aminoethyl)cysteine, or are

auxotrophic for regulatorily significant metabolites and produce L-amino acids, such as for example L-lysine.

For some years, methods of recombinant DNA technology have likewise been used to improve strains of *Corynebacterium* which produce amino acids by amplifying individual biosynthesis genes and investigating the effect on amino acid production. Review articles on this subject may be found inter alia in Kinoshita ("Glutamic Acid Bacteria", in: *Biology of Industrial Microorganisms*, Demain and Solomon (Eds.), Benjamin Cummings, London, UK, 1985, 115-142), Hilliger (*BioTec* 2, 40-44 (1991)), Eggeling (*Amino Acids* 6:261-272 (1994)), Jetten and Sinskey (*Critical Reviews in Biotechnology* 15, 73-103 (1995)) and Sahm et al. (*Annals of the New York Academy of Science* 782, 25-39 (1996)).

SUMMARY OF THE INVENTION

Object of the invention

It is an object of the invention to provide novel means for the improved fermentative production of amino acids, in particular L-lysine.

Description of the invention

Amino acids, in particular L-lysine, are used in human medicine, in the pharmaceuticals industry and in particular in animal nutrition. There is accordingly general interest in providing novel improved processes for the production of amino acids, in particular L-lysine.

Any subsequent mention of L-lysine or lysine should be understood to mean not only the base, but also salts, such as for example lysine monohydrochloride or lysine sulfate.

The invention provides an isolated polynucleotide from coryneform bacteria containing a polynucleotide sequence selected from the group

- a) a polynucleotide which is at least 70% identical to a polynucleotide which encodes a polypeptide containing the amino acid sequence of SEQ ID NO:2,
- 5 b) a polynucleotide which encodes a polypeptide which contains an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID NO:2,
- c) a polynucleotide which is complementary to the polynucleotides of a) or b), and
- 10 d) polynucleotide containing at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c).

The present invention also provides a polynucleotide with the features noted above, and comprising replicable DNA containing:

- 15 (i) the nucleotide sequence shown in SEQ ID NO:1, or
- (ii) at least one sequence which matches sequence (i) within the degeneration range of the genetic code, or
- (iii) at least one sequence which hybridises with the
- 20 complementary sequence to sequence (i) or (ii) and optionally
- (iv) functionally neutral sense mutations in (i).

The present invention also provides

- a polynucleotide with the aforementioned features,
- 25 containing the nucleotide sequence as shown in SEQ ID NO:1,

a polynucleotide that is a replicable DNA with feature(s) of a)-d) that encodes a polypeptide which contains the amino acid sequence as shown in SEQ ID NO:2,

a vector containing the polynucleotide with feature(s) of a)-d), in particular a shuttle vector or plasmid vector and coryneform bacteria acting as host cell which contain the vector.

- 5 The present invention also provides polynucleotides which substantially consist of a polynucleotide sequence, which are obtainable by screening by means of hybridisation of a suitable gene library, which contains the complete gene having the polynucleotide sequence according to SEQ ID
10 NO:1, with a probe which contains the sequence of the stated polynucleotide according to SEQ ID NO:1, or a fragment thereof, and isolation of the stated DNA sequence.

Polynucleotide sequences according to the invention are suitable as hybridisation probes for RNA, cDNA and DNA in
15 order to isolate full length cDNA which code for phosphofructokinase and to isolate such cDNA or genes, the sequence of which exhibits a high level of similarity with that of the phosphofructokinase gene.

Polynucleotide sequences according to the invention are
20 furthermore suitable as primers for the production of DNA of genes which code for phosphofructokinase by the polymerase chain reaction (PCR).

Such oligonucleotides acting as probes or primers contain at least 30, preferably at least 20, very particularly
25 preferably at least 15 successive nucleotides. Oligonucleotides having a length of at least 40 or 50 nucleotides are also suitable.

"Isolated" means separated from its natural environment.

"Polynucleotide" generally relates to polyribonucleotides
30 and polydeoxyribonucleotides, wherein the RNA or DNA may be unmodified or modified.

"Polypeptides" are taken to mean peptides or proteins which contain two or more amino acids connected by peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID NO:2, in particular those
5 having the biological activity of phosphofructokinase and also those which are at least 70%, preferably at least 80%, identical to the polypeptide according to SEQ ID NO:2 and in particular are 90% to 95% identical to the polypeptide according to SEQ ID NO:2 and exhibit the stated activity.

10 The invention furthermore relates to a process for the fermentative production of amino acids, in particular L-lysine, using coryneform bacteria, which in particular already produce an amino acid and in which the nucleotide sequences which code for the pfkA gene are amplified, in
15 particular overexpressed.

In this connection, the term "amplification" describes the increase in the intracellular activity of one or more enzymes in a microorganism, which enzymes are encoded by the corresponding DNA, for example by increasing the copy
20 number of the gene or genes, by using a strong promoter or a gene which encodes a corresponding enzyme having elevated activity and optionally by combining these measures.

The microorganisms provided by the present invention may produce L-amino acids, in particular L-lysine, from
25 glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. The microorganisms may comprise representatives of the coryneform bacteria in particular of the genus *Corynebacterium*. Within the genus *Corynebacterium*, the
30 species *Corynebacterium glutamicum* may in particular be mentioned, which is known in specialist circles for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum*, are for example the known wild type strains

- 5 *Corynebacterium glutamicum* ATCC13032
 Corynebacterium acetoglutamicum ATCC15806
 Corynebacterium acetoacidophilum ATCC13870
 Corynebacterium thermoaminogenes FERM BP-1539
 Corynebacterium melassecola ATCC17965
 Brevibacterium flavum ATCC14067
10 *Brevibacterium lactofermentum* ATCC13869 and
 Brevibacterium divaricatum ATCC14020

and L-lysine producing mutants or strains produced therefrom, such as for example

- 15 *Corynebacterium glutamicum* FERM-P 1709
 Brevibacterium flavum FERM-P 1708
 Brevibacterium lactofermentum FERM-P 1712
 Corynebacterium glutamicum FERM-P 6463
 Corynebacterium glutamicum FERM-P 6464 and
 Corynebacterium glutamicum DSM5715.

- 20 The inventors succeeded in isolating the novel *pfkA* gene, which codes for the enzyme phosphofructokinase (EC 2.7.1.11), from *C. glutamicum*.

- 25 The *pfkA* gene and other genes from *C. glutamicum* are isolated by initially constructing a gene library of this microorganism in *E. coli*. The construction of gene libraries is described in generally known textbooks and manuals. Examples which may be mentioned are the textbook by Winnacker, Gene und Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990) or
30 the manual by Sambrook et al., Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). One very well known gene library is that of *E. coli* K-12 strain W3110, which was constructed by Kohara et al.

(Cell 50, 495-508 (1987)) in λ -vectors. Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which was constructed using the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in *E. coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575). Börmann et al. (Molecular Microbiology 6(3), 317-326, 1992)) also describe a gene library of *C. glutamicum* ATCC 13032, using cosmid pH79 (Hohn and Collins, Gene 11, 291-298 (1980)). A gene library of *C. glutamicum* in *E. coli* may also be produced using plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are in particular those *E. coli* strains with restriction and recombination defects. One example of such a strain is the strain DH5 α mcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the assistance of cosmids may then in turn be sub-cloned in usual vectors suitable for sequencing and then be sequenced, as described, for example, in Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The novel DNA sequence from *C. glutamicum* that encodes the *pfkA* gene and is provided by the present invention as SEQ ID NO:1 was obtained in this manner. The amino acid sequence of the corresponding protein was furthermore deduced from the above DNA sequence using the methods described above. SEQ ID NO:2 shows the resultant amino acid sequence of the product of the *pfkA* gene.

Coding DNA sequences arising from the degeneracy of the genetic code are also provided by the present invention. DNA sequences which hybridise with SEQ ID NO:1 or portions of SEQ ID NO:1 are also provided by the invention.

Conservative substitutions of amino acids in proteins, for example the substitution of glycine for alanine or of aspartic acid for glutamic acid, are known in specialist circles as "sense mutations", which result in no

5 fundamental change in activity of the protein, i.e. they are functionally neutral. It is furthermore known that changes to the N and/or C terminus of a protein do not substantially impair or may even stabilise the function thereof. The person skilled in the art will find

10 information in this connection inter alia in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in other standard

15 textbooks of genetics and molecular biology. Amino acid sequences arising in a corresponding manner from SEQ ID NO:2 are also provided by the present invention.

Similarly, DNA sequences which hybridise with SEQ ID NO:1 or portions of SEQ ID NO:1 are also provided by the present

20 invention. Finally, DNA sequences produced by the polymerase chain reaction (PCR) using primers obtained from SEQ ID NO:1 are also provided by the present invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

25 The person skilled in the art may find instructions for identifying DNA sequences by means of hybridisation inter alia in the manual "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal

30 of Systematic Bacteriology (1991) 41: 255-260). The person skilled in the art may find instructions for amplifying DNA sequences using the polymerase chain reaction (PCR) inter alia in the manual by Gait, Oligonucleotide synthesis: a practical approach (IRL Press, Oxford, UK, 1984) and in

Newton & Graham, PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

The inventors discovered that coryneform bacteria produce L-amino acids, in particular L-lysine, in an improved
5 manner once the pfkA has been overexpressed.

Overexpression may be achieved by increasing the copy number of the corresponding genes or by mutating the promoter and regulation region or the ribosome-binding site located upstream from the structural gene. Expression
10 cassettes incorporated upstream from the structural gene act in the same manner. It is additionally possible to increase expression during fermentative L-lysine production by means of inducible promoters. Expression is also improved by measures to extend the lifetime of the mRNA.
15 Enzyme activity is moreover amplified by preventing degradation of the enzyme protein. The genes or gene constructs may either be present in plasmids in a variable copy number or be integrated in the chromosome and amplified. Alternatively, overexpression of the genes
20 concerned may also be achieved by modifying the composition of the nutrient media and culture conditions.

The person skilled in the art will find guidance in this connection *inter alia* in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41
25 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in European patent EP 0 472 869, in US patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991), in Reinscheid et al. (Applied and Environmental Microbiology
30 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in patent application WO 96/15246, in Malumbres et al. (Gene 134, 15-24 (1993)), in Japanese published patent application JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58,
35 191-195 (1998)), in Makrides (Microbiological Reviews

60:512-538 (1996)) and in known textbooks of genetics and molecular biology.

By way of example, the pfkA gene according to the invention was overexpressed with the assistance of plasmids.

5 Suitable plasmids are those which are replicated in coryneform bacteria. Numerous known plasmid vectors, such as for example pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen
10 et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as for example those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), or pAG1 (US-A 5,158,891) may be used in
15 the same manner.

Further suitable plasmid vectors are those with the assistance of which gene amplification may be performed by integration into the chromosome, as has for example been described by Reinscheid et al. (Applied and Environmental
20 Microbiology 60, 126-132 (1994)) for the duplication or amplification of the hom-thrB operon. In this method, the complete gene is cloned into a plasmid vector which can replicate in a host (typically E. coli), but not in C. glutamicum. Vectors which may be considered are, for
25 example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-A 5,487,993), pCR®Blunt (Invitrogen,
30 Groningen, Netherlands; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516). The plasmid vector which contains the gene to be amplified is then transferred into the desired strain of C. glutamicum
35 by conjugation or transformation. The conjugation method is

described, for example, in Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)).

Transformation methods are described, for example, in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of "crossing over", the resultant strain contains at least two copies of the gene in question.

It may additionally be advantageous for the production of amino acids, in particular L-lysine, to amplify or overexpress not only the pfkA gene, but also one or more enzymes of the particular biosynthetic pathway, of glycolysis, of anaplerotic metabolism, of the citric acid cycle or of amino acid export.

For the production of L-lysine, for example, it is thus possible simultaneously to overexpress one or more genes selected from the group

- the dapA gene which encodes dihydropicolinate synthase (EP-B 0 197 335), or
- the gap gene which encodes glyceraldehyde-3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086), or
- the tpi gene which encodes triosephosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086), or
- the pgk gene which encodes 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086), or
- the pyc gene which encodes pyruvate carboxylase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086), or

- the lysE gene which codes for lysine export (DE-A-195 48 222).

It may furthermore be advantageous for the production of amino acids, in particular L-lysine, in addition to
5 amplifying the pfkA gene, simultaneously to attenuate

- the pck gene which encodes phosphoenolpyruvate carboxykinase (DE 199 50 409.1, DSM 13047) and/or
- the pgi gene which encodes glucose 6-phosphate isomerase (US 09/396,478, DSM 12969).

10 It may furthermore be advantageous for the production of amino acids, in particular L-lysine, in addition to overexpressing the pfkA gene, to suppress unwanted secondary reactions (Nakayama: "Breeding of Amino Acid
Producing Micro-organisms", in: Overproduction of Microbial
15 Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

For the purposes of amino acid production, in particular of L-lysine, the microorganisms produced according to the invention may be cultured continuously or discontinuously
20 using the batch process or the fed batch process or repeated fed batch process. A summary of known culture methods is given in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart,
25 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must adequately satisfy the requirements of the particular strains. Culture media for
30 various microorganisms are described in "Manual of Methods for General Bacteriology" from the American Society for Bacteriology (Washington D.C., USA, 1981). Carbon sources

which may be used are sugars and carbohydrates, such as glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose for example, oils and fats, such as soya oil, sunflower oil, peanut oil and coconut oil for example, fatty acids, such as palmitic acid, stearic acid and linoleic acid for example, alcohols, such as glycerol and ethanol for example, and organic acids, such as acetic acid for example. These substances may be used individually or as a mixture. Nitrogen sources which may be used comprise organic compounds containing nitrogen, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya flour and urea or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or as a mixture. Phosphorus sources which may be used are phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding salts containing sodium. The culture medium has additionally to contain salts of metals, such as magnesium sulfate or iron sulfate for example, which are necessary for growth. Finally, essential growth-promoting substances such as amino acids and vitamins may also be used in addition to the above-stated substances. Suitable precursors may furthermore be added to the culture medium. The stated feed substances may be added to the culture as a single batch or be fed appropriately during culturing.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds, such as phosphoric acid or sulfuric acid, are used appropriately to control the pH of the culture. Foaming may be controlled by using antifoaming agents such as fatty acid polyglycol esters for example. Plasmid stability may be maintained by the addition to the medium of suitable selectively acting substances, for example antibiotics. Oxygen or oxygen-containing gas mixtures, such as air for

example, are introduced into the culture in order to maintain aerobic conditions. The temperature of the culture is normally from 20°C to 45°C and preferably from 25°C to 40°C. The culture is continued until the maximum quantity
5 of lysine has formed. This aim is normally achieved within 10 to 160 hours.

Analysis of L-lysine may be performed by anion exchange chromatography with subsequent ninhydrin derivatisation, as described in Spackman et al. (Analytical Chemistry, 30,
10 (1958), 1190).

The purpose of the process according to the invention is the fermentative production of amino acids, in particular L-lysine.

15 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Map of plasmid pT-pfkAexp

Figure 2: Map of plasmid pT-pfkAexp

The abbreviations and names are defined as follows.

20	Tet:	Resistance gene for tetracycline
	oriV:	Plasmid-code replication origin of E. coli
	RP4mob:	mob region for plasmid mobilisation
	rep:	Plasmid-coded replication origin from 25 C. glutamicum plasmid pGA1
	per:	Gene for controlling copy number from pGA1
	lacZ-alpha:	lacZ α gene fragment (N terminus) of the β -galactosidase gene
	'lacZa':	5' end of the lacZ α gene fragment
30	lacZ-alpha':	3' end of the lacZ α gene fragment
	pfkA:	pfkA gene from C. glutamicum ATCC13032
	BamHI:	Restriction site of the restriction enzyme BamHI
	EcoRI:	Restriction site of the restriction enzyme

EcoRI
HindIII: Restriction site of the restriction enzyme
HindIII
KpnI: Restriction site of the restriction enzyme
5 KpnI
PstI: Restriction site of the restriction enzyme
PstI
PvuI: Restriction site of the restriction enzyme
PvuI
10 SalI: Restriction site of the restriction enzyme
SalI
SacI: Restriction site of the restriction enzyme
SacI
SmaI: Restriction site of the restriction enzyme
15 SmaI
SphI: Restriction site of the restriction enzyme
SphI
XbaI: Restriction site of the restriction enzyme
XbaI
20 XhoI: Restriction site of the restriction enzyme
XhoI

DETAILED DESCRIPTION OF THE INVENTION

25

The present invention is illustrated in greater detail by the following practical examples.

Example 1

30 Production of a genomic cosmid gene library from
Corynebacterium glutamicum ATCC13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032
was isolated as described in Tauch et al., (1995, Plasmid
33:168-179) and partially cleaved with the restriction
35 enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany,

product description Sau3AI, code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, product description SAP, code no. 1758250). The

5 DNA of cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), purchased from Stratagene (La Jolla, USA, product description SuperCos1 Cosmid Vector Kit, code no. 251301) was cleaved with the restriction enzyme XbaI

10 (Amersham Pharmacia, Freiburg, Germany, product description XbaI, code no. 27-0948-02) and also dephosphorylated with shrimp alkaline phosphatase. The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI,

15 code no. 27-0868-04). Cosmid DNA treated in this manner was mixed with the treated ATCC 13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, product description T4 DNA Ligase, code no. 27-0870-04). The ligation mixture was then packed in phages

20 using Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, product description Gigapack II XL Packing Extract, code no. 200217). E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Res. 16:1563-1575) was infected by suspending the cells in 10 mM MgSO₄ and mixing them with an

25 aliquot of the phage suspension. The cosmid library was infected and titred as described in Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100µg/ml of ampicillin. After

30 overnight incubation at 37°C, individual recombinant clones were selected.

Example 2

Isolation and sequencing of the pfkA gene

Cosmid DNA from an individual colony was isolated in accordance with the manufacturer's instructions using the Qiaprep Spin Miniprep Kit (product no. 27106, Qiagen, Hilden, Germany) and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, product no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, product description SAP, product no. 1758250).

Once separated by gel electrophoresis, the cosmid fragments of a size of 1500 to 2000 bp were isolated using the QiaExII Gel Extraction Kit (product no. 20021, Qiagen, Hilden, Germany). The DNA of the sequencing vector pZero-1 purchased from Invitrogen (Groningen, Netherlands, product description Zero Background Cloning Kit, product no. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, product no. 27-0868-04). Ligation of the cosmid fragments into the sequencing vector pZero-1 was performed as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated into the E. coli strain DH5 α MCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) and plated out onto LB agar (Lennox, 1955, Virology, 1:190) with 50 μ g/ml of Zeocin. Plasmids of the recombinant clones were prepared using the Biorobot 9600 (product no. 900200, Qiagen, Hilden, Germany). Sequencing was performed using the dideoxy chain termination method according to Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) as modified by Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (product no. 403044, Weiterstadt, Germany) was used.

Separation by gel electrophoresis and analysis of the sequencing reaction was performed in a "Rotiphorese NF" acrylamide/bisacrylamide gel (29:1) (product no. A124.1, Roth, Karlsruhe, Germany) using the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The resultant raw sequence data were then processed using the Staden software package (1986, Nucleic Acids Research, 14:217-231), version 97-0. The individual sequences of the pZero 1 derivatives were assembled into a cohesive contig. Computer-aided coding range analysis was performed using XNIP software (Staden, 1986, Nucleic Acids Research, 14:217-231). Further analysis was performed using the "BLAST search programs" (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402), against the non-redundant database of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The resultant nucleotide sequence is shown as SEQ ID NO:1. Analysis of the nucleotide sequence revealed an open reading frame of 1029 base pairs, which was designated the pfkA gene. The pfkA gene encodes a protein of 343 amino acids.

Example 3

Production of a plasmid for overexpressing pfkA in *Corynebacterium glutamicum*

3.1. Cloning of pfkA in the pCR2-Blunt vector

Chromosomal DNA from *Corynebacterium glutamicum* ATCC 13032 was isolated as described in Tauch et al., (1995, Plasmid 33:168-179). On the basis of the sequence of the pfkA gene for *C. glutamicum* known from Example 2, the following oligonucleotides were selected for the polymerase chain reaction:

pfkA-exp

5`-AAC TGC AGC TCT GGC GAT TA-3`

pfk-ex2

5`-AAC TAT CCA AAC ATT GCC TG-3`

- 5 These primers were synthesised by the company MWG Biotech (Ebersberg, Germany) and the PCR reaction performed in accordance with the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) using Pwo polymerase from Roche Diagnostics GmbH (Mannheim, Germany). A DNA fragment of approx. 1160 bp in size, which bears the pfkA gene, was isolated with the assistance of the polymerase chain reaction.

- The amplified DNA fragment was ligated into the vector pCR2 Blunt Vector (Bernard et al., (1983) Journal of Molecular Biology. 234:534-541) using the Zero Blunt PCR Cloning Kit from Invitrogen Corporation (Carlsbad, CA, USA; catalogue number K2700-20). The E. coli strain Top10F (Grand et al. (1990) Proceedings of the National Academy of Sciences, USA. 87:4645-4649) was transformed with the ligation batch.
- 20 Plasmid-bearing cells were selected by plating the transformation batch out onto LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) which had been supplemented with 50 mg/l of kanamycin. Plasmid
- 25 DNA was isolated from a transformant using the QIAprep Spin Miniprep Kit from Qiagen and verified by restriction with the restriction enzyme EcoRI and subsequent agarose gel electrophoresis (0.8%). The plasmid was named pCRB1-pfkAexp1.

30

3.2. Production of the shuttle vector pEC-T18mob2

The E. coli-C. glutamicum shuttle vector was constructed in accordance with the prior art. The vector contains the

replication region rep of plasmid pGA1, including the replication effector per (U.S. Pat. No. 5,175,108; Nesvera et al., Journal of Bacteriology 179, 1525-1532 (1997)), the tetA(Z) gene, which imparts tetracycline resistance, of
5 plasmid pAG1 (U.S. Pat. No. 5,158,891; gene library entry at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) with the accession number AF121000), the replication origin oriV of plasmid pMB1 (Sutcliffe, Cold Spring Harbor Symposium on Quantitative Biology 43, 77-90
10 (1979)), the lacZ α gene fragment including the lac promoter and a multiple cloning site (mcs) (Norranders et al. Gene 26, 101-106 (1983)) and the mob region of plasmid RP4 (Simon et al., (1983) Bio/Technology 1:784-791). The constructed vector was then transformed into E. coli strain
15 DH5 α (Hanahan, in: DNA cloning. A practical approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Plasmid-bearing cells were selected by plating the transformation batch out onto LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory
20 Press, Cold Spring Harbor, N.Y.) which had been supplemented with 5 mg/l of tetracycline. Plasmid DNA was isolated from a transformant using the QIAprep Spin Miniprep Kit from Qiagen and verified by restriction with the restriction enzymes EcoRI and HindIII and subsequent
25 agarose gel electrophoresis (0.8%). The plasmid was named pEC-T18mob2 and is shown in Figure 1.

3.3. Cloning of pfkA into shuttle vector pEC-T18mob2

The vector used was the E. coli-C. glutamicum shuttle
30 vector pEC-T18mob2 described in Example 3.2. DNA from this plasmid was completely cleaved with the restriction enzyme EcoRI and then dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, product description SAP, product no. 1758250).

The pfkA gene was isolated from the plasmid pCRB1-pfkAexp1 described in Example 3.1. by complete cleavage with the enzyme EcoRI. The approx. 1160 bp pfkA fragment was isolated from the agarose gel using the QiaExII Gel
5 Extraction Kit (product no. 20021, Qiagen, Hilden, Germany).

The pfkA fragment obtained in this manner was mixed with the prepared pEC-T18mob2 vector and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany,
10 product description T4 DNA Ligase, code no. 27-0870-04). The ligation batch was then transformed into E. coli strain DH5 α (Hanahan, in: DNA cloning. A practical approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Plasmid-bearing cells were selected by plating the transformation batch out
15 onto LB agar (Lennox, 1955, Virology, 1:190) with 5 mg/l of tetracycline. After overnight incubation at 37°C, individual recombinant clones were selected. Plasmid DNA was isolated from a transformant in accordance with the manufacturer's instructions using the Qiaprep Spin Miniprep
20 Kit (product no. 27106, Qiagen, Hilden, Germany) and cleaved with the restriction enzyme EcoRI in order to check the plasmid by subsequent agarose gel electrophoresis. The resultant plasmid was named pT-pfkAexp. It is shown in Figure 2.

25

Example 4:

Transformation of strain DSM5715 with plasmid pT-pfkAexp

Strain DSM5715 (EP-B-0 435 132) was then transformed with plasmid pT-pfkAexp using the electroporation method
30 described by Liebl et al. (FEMS Microbiology Letters, 53:299-303 (1989)). Transformant selection proceeded on LBHIS agar consisting of 18.5 g/l of brain-heart infusion bouillon, 0.5 M sorbitol, 5 g/l of Bacto tryptone, 2.5 g/l

of Bacto yeast extract, 5 g/l of NaCl and 18 g/l of Bacto agar, which had been supplemented with 5 mg/l of tetracycline. Incubation was performed for 2 days at 33°C.

5 Plasmid DNA was isolated from a transformant using the conventional methods (Peters-Wendisch et al., 1998, Microbiology, 144, 915 - 927), cut with the restriction endonuclease EcoRI in order to check the plasmid by subsequent agarose gel electrophoresis. The resultant strain was named DSM5715/pT-pfkAexp.

10

Example 5

Production of lysine

15 The *C. glutamicum* strain DSM5715/pT-pfkAexp obtained in Example 4 was cultured in a nutrient medium suitable for the production of lysine and the lysine content of the culture supernatant was determined.

To this end, the strain was initially incubated for 24 hours at 33°C on an agar plate with the appropriate antibiotic (brain/heart agar with tetracycline (5 mg/l)).

20 Starting from this agar plate culture, a preculture was inoculated (10 ml of medium in a 100 ml Erlenmeyer flask). The medium used for the preculture was complete medium CgIII (2.5 g/l of NaCl, 10 g/l of Bacto peptone, 10 g/l of Bacto yeast extract, 20 g/l of glucose, pH 7.4).

25 Tetracycline (5 mg/l) was added to this medium. The preculture was incubated for 16 hours at 33°C on a shaker at 240 rpm. A main culture was inoculated from this preculture, such that the initial OD (660 nm) of the main culture was 0.1. Medium MM was used for the main culture.

Medium MM

CSL (Corn Steep Liquor)	5 g/l
MOPS (Morpholinopropanesulfonic acid)	20 g/l
Glucose (separately autoclaved)	50 g/l
$(\text{NH}_4)_2\text{SO}_4$	25 g/l
KH_2PO_4	0.1 g/l
$\text{MgSO}_4 * 7 \text{ H}_2\text{O}$	1.0 g/l
$\text{CaCl}_2 * 2 \text{ H}_2\text{O}$	10 mg/l
$\text{FeSO}_4 * 7 \text{ H}_2\text{O}$	10 mg/l
$\text{MnSO}_4 * \text{H}_2\text{O}$	5.0 mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-leucine (sterile-filtered)	0.1 g/l
CaCO_3	25 g/l

CSL, MOPS and the salt solution were adjusted to pH 7 with ammonia water and autoclaved. The sterile substrate and vitamin solutions, together with the dry-autoclaved CaCO_3 are then added.

Culturing is performed in a volume of 10 ml in a 100 ml Erlenmeyer flask with flow spoilers. Kanamycin (25 mg/l) was added. Culturing was performed at 33°C and 80% atmospheric humidity.

After 24 hours, the OD was determined at a measurement wavelength of 660 nm using a Biomek 1000 (Beckmann Instruments GmbH, Munich). The quantity of lysine formed was determined using an amino acid analyser from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatisation with ninhydrin detection.

Table 1 shows the result of the test.

Table 1

Strain	OD(660)	Lysine HCl g/l
DSM5715/pT-pfkAexp	14.6	10.1
DSM5715	15.2	8.1

SEQUENCE LISTING

<110> Degussa-Hüls AG

5 <120> Novel nucleotide sequences encoding the pfkA gene

<130> 990169 BT

<140>

10 <141>

<160> 2

<170> PatentIn Ver. 2.1

15

<210> 1

<211> 1274

<212> DNA

<213> Corynebacterium glutamicum

20

<220>

<221> CDS

<222> (143)..(1171)

25

<400> 1

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ttggcctgtc aacccctgtg attctcttat ttttgggtga ttgttcgggc gcgggtgttg 120

30

tgatgggttt aatatggaag ac atg cga att gct act ctc acg tca ggc ggc 172

Met Arg Ile Ala Thr Leu Thr Ser Gly Gly

1

5

10

35

gac tgc ccc gga cta aac gcc gtc atc cga gga atc gtc cgc aca gcc 220

Asp Cys Pro Gly Leu Asn Ala Val Ile Arg Gly Ile Val Arg Thr Ala

15

20

25

40

agc aat gaa ttt ggc tcc acc gtc gtt ggt tat caa gac ggt tgg gaa 268

Ser Asn Glu Phe Gly Ser Thr Val Val Gly Tyr Gln Asp Gly Trp Glu

30

35

40

45

gga ctg tta ggc gat cgt cgc gta cag ctg tat gac gat gaa gat att 316

Gly Leu Leu Gly Asp Arg Arg Val Gln Leu Tyr Asp Asp Glu Asp Ile

45

50

55

gac cga atc ctc ctt cga ggc ggc acc att ttg ggc act ggt cgc ctc 364

Asp Arg Ile Leu Leu Arg Gly Gly Thr Ile Leu Gly Thr Gly Arg Leu

60

65

70

50

cat ccg gac aag ttt aag gcc gga att gat cag att aag gcc aac tta 412

His Pro Asp Lys Phe Lys Ala Gly Ile Asp Gln Ile Lys Ala Asn Leu

75

80

85

90

55

gaa gac gcc ggc atc gat gcc ctt atc cca atc ggt ggc gaa gga acc 460

Glu Asp Ala Gly Ile Asp Ala Leu Ile Pro Ile Gly Gly Glu Gly Thr

95

100

105

	ctg aag ggt gcc aag tgg ctg tct gat aac ggt atc cct gtt gtc ggt	508
	Leu Lys Gly Ala Lys Trp Leu Ser Asp Asn Gly Ile Pro Val Val Gly	
	110 115 120	
5	gtc cca aag acc att gac aat gac gtg aat ggc act gac ttc acc ttc	556
	Val Pro Lys Thr Ile Asp Asn Asp Val Asn Gly Thr Asp Phe Thr Phe	
	125 130 135	
10	ggc ttc gat act gct gtg gca gtg gct acc gac gct gtt gac cgc ctg	604
	Gly Phe Asp Thr Ala Val Ala Val Ala Thr Asp Ala Val Asp Arg Leu	
	140 145 150	
15	cac acc acc gct gaa tct cac aac cgt gtg atg atc gtg gag gtc atg	652
	His Thr Thr Ala Glu Ser His Asn Arg Val Met Ile Val Glu Val Met	
	155 160 165 170	
20	ggc cgc cac gtg ggt tgg att gct ctg cac gca ggt atg gcc ggc ggt	700
	Gly Arg His Val Glu Trp Ile Ala Leu His Ala Gly Met Ala Gly Gly	
	175 180 185	
25	gct cac tac acc gtt att cca gaa gta cct ttc gat att gca gag atc	748
	Ala His Tyr Thr Val Ile Pro Glu Val Pro Phe Asp Ile Ala Glu Ile	
	190 195 200	
30	tgc aag gcg atg gaa cgt cgc ttc cag atg ggc gag aag tac ggc att	796
	Cys Lys Ala Met Glu Arg Arg Phe Gln Met Gly Glu Lys Tyr Gly Ile	
	205 210 215	
35	atc gtc gtt gcg gaa ggt gcg ttg cca cgc gaa ggc acc atg gag ctt	844
	Ile Val Val Ala Glu Gly Ala Leu Pro Arg Glu Gly Thr Met Glu Leu	
	220 225 230	
40	cgt gaa ggc cac att gac cag ttc ggt cac aag acc ttc acg gga att	892
	Arg Glu Gly His Ile Asp Gln Phe Gly His Lys Thr Phe Thr Gly Ile	
	235 240 245 250	
45	gga cag cag atc gct gat gag atc cac gtg cgc ctc ggc cac gat gtt	940
	Gly Gln Gln Ile Ala Asp Glu Ile His Val Arg Leu Gly His Asp Val	
	255 260 265	
50	cgt acg acc gtt ctt ggc cac att caa cgt ggt gga acc cca act gct	988
	Arg Thr Thr Val Leu Gly His Ile Gln Arg Gly Gly Thr Pro Thr Ala	
	270 275 280	
55	ttc gac cgt gtt ctg gcc act cgt tat ggt gtt cgt gca gct cgt gcg	1036
	Phe Asp Arg Val Leu Ala Thr Arg Tyr Gly Val Arg Ala Ala Arg Ala	
	285 290 295	
60	tgc cat gag gga agc ttt gac aag gtt gtt gct ttg aag ggt gag agc	1084
	Cys His Glu Gly Ser Phe Asp Lys Val Val Ala Leu Lys Gly Glu Ser	
	300 305 310	
65	att gag atg atc acc ttt gaa gaa gca gtc gga acc ttg aag gaa gtt	1132
	Ile Glu Met Ile Thr Phe Glu Glu Ala Val Gly Thr Leu Lys Glu Val	
	315 320 325 330	

cca ttc gaa cgc tgg gtt act gcc cag gca atg ttt gga tagtttttcg 1181
 Pro Phe Glu Arg Trp Val Thr Ala Gln Ala Met Phe Gly
 335 340

5

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catgccgtaa ggaaagtgca agtaagtgaa atc 1274

10

<210> 2

<211> 343

<212> PRT

<213> Corynebacterium glutamicum

15

<400> 2

Met Arg Ile Ala Thr Leu Thr Ser Gly Gly Asp Cys Pro Gly Leu Asn
 1 5 10 15

20

Ala Val Ile Arg Gly Ile Val Arg Thr Ala Ser Asn Glu Phe Gly Ser
 20 25 30

Thr Val Val Gly Tyr Gln Asp Gly Trp Glu Gly Leu Leu Gly Asp Arg
 35 40 45

25

Arg Val Gln Leu Tyr Asp Asp Glu Asp Ile Asp Arg Ile Leu Leu Arg
 50 55 60

30

Gly Gly Thr Ile Leu Gly Thr Gly Arg Leu His Pro Asp Lys Phe Lys
 65 70 75 80

Ala Gly Ile Asp Gln Ile Lys Ala Asn Leu Glu Asp Ala Gly Ile Asp
 85 90 95

35

Ala Leu Ile Pro Ile Gly Gly Glu Gly Thr Leu Lys Gly Ala Lys Trp
 100 105 110

Leu Ser Asp Asn Gly Ile Pro Val Val Gly Val Pro Lys Thr Ile Asp
 115 120 125

40

Asn Asp Val Asn Gly Thr Asp Phe Thr Phe Gly Phe Asp Thr Ala Val
 130 135 140

45

Ala Val Ala Thr Asp Ala Val Asp Arg Leu His Thr Thr Ala Glu Ser
 145 150 155 160

His Asn Arg Val Met Ile Val Glu Val Met Gly Arg His Val Gly Trp
 165 170 175

50

Ile Ala Leu His Ala Gly Met Ala Gly Gly Ala His Tyr Thr Val Ile
 180 185 190

Pro Glu Val Pro Phe Asp Ile Ala Glu Ile Cys Lys Ala Met Glu Arg
 195 200 205

55

Arg Phe Gln Met Gly Glu Lys Tyr Gly Ile Ile Val Val Ala Glu Gly
 210 215 220

Ala Leu Pro Arg Glu Gly Thr Met Glu Leu Arg Glu Gly His Ile Asp
225 230 235 240

5 Gln Phe Gly His Lys Thr Phe Thr Gly Ile Gly Gln Gln Ile Ala Asp
245 250 255

Glu Ile His Val Arg Leu Gly His Asp Val Arg Thr Thr Val Leu Gly
260 265 270

10 His Ile Gln Arg Gly Gly Thr Pro Thr Ala Phe Asp Arg Val Leu Ala
275 280 285

15 Thr Arg Tyr Gly Val Arg Ala Ala Arg Ala Cys His Glu Gly Ser Phe
290 295 300

Asp Lys Val Val Ala Leu Lys Gly Glu Ser Ile Glu Met Ile Thr Phe
305 310 315 320

20 Glu Glu Ala Val Gly Thr Leu Lys Glu Val Pro Phe Glu Arg Trp Val
325 330 335

Thr Ala Gln Ala Met Phe Gly
340

25

WHAT IS CLAIMED IS:

1. Isolated polynucleotide from coryneform bacteria
containing a polynucleotide sequence selected from the
5 group consisting of
 - a) a polynucleotide which is at least 70% identical
to a polynucleotide which encodes a polypeptide
containing the amino acid sequence of SEQ ID NO:
2,
 - 10 b) a polynucleotide which encodes a polypeptide
which contains an amino acid sequence which is at
least 70% identical to the amino acid sequence of
SEQ ID NO:2,
 - c) a polynucleotide which is complementary to the
15 polynucleotides of a) or b), and
 - e) a polynucleotide containing at least 15
successive bases of the polynucleotide sequence
of a), b) or c).
2. The polynucleotide according to claim 1,
20 wherein the polynucleotide is DNA replicable in
coryneform bacteria.
3. The polynucleotide according to claim 2 which is
recombinant DNA.
4. The polynucleotide according to claim 1,
25 wherein the polynucleotide is an RNA.
5. The polynucleotide according to claim 2,
containing the nucleic acid sequence represented in SEQ
ID NO:1.
6. The replicable DNA according to claim 2,
30 containing

- (i) the nucleotide sequence shown in SEQ ID NO:1, or
 - (ii) at least one sequence which matches the sequence (i) within the degeneration range of the genetic code, or
 - 5 (iii) at least one sequence which hybridises with the complementary sequence to sequence (i) or (ii) and optionally
 - (iv) functionally neutral sense mutations in (i).
- 10 7. The polynucleotide sequence according to claim 2 which encodes a polypeptide which contains the amino acid sequence shown in SEQ ID NO:2.
8. A process for the fermentative production of L-amino acids, in particular L-lysine, comprising the following steps:
- 15 a) fermentation of L-amino acid producing coryneform bacteria in which at least the pfkA gene or nucleotide sequences coding therefor is/are amplified,
- 20 b) accumulation of the L-amino acid in the medium or in the cells of the bacteria and
- c) isolation of the L-amino acid.
9. The process according to claim 8 wherein the gene or sequences are amplified by overexpression.
- 25 10. The process according to claim 8, wherein bacteria are used in which further genes of the biosynthetic pathway of the desired L-amino acid are additionally amplified.
- 30 11. The process according to claim 8, wherein bacteria are used in which the metabolic pathways which reduce the formation of L-lysine are at least partially suppressed.

12. The process according to claim 8, wherein
a strain transformed with a plasmid vector is used and
the plasmid vector bears the nucleotide sequences
which encode the pfkA gene.
- 5 13. The process according to one of claims 8 to 12,
wherein coryneform bacteria are used which produce L-
lysine.
- 10 14. The process according to claim 8, wherein
bacteria are fermented for the production of lysine in
which one or more of the genes selected from the group
- a) the dapA gene which encodes dihydropicolinate
synthase,
- b) the pyc gene, which encodes pyruvate carboxylase,
- 15 c) the tpi gene, which encodes triosephosphate
isomerase,
- d) the dapE gene, which encodes
succinyldiaminopimelate desuccinylase,
- e) the gap gene, which encodes glyceraldehyde
3-phosphate dehydrogenase,
- 20 f) the pgk gene, which encodes 3-phosphoglycerate
kinase, and
- g) the lysE gene, which encodes for lysine export,
is/are simultaneously amplified.
- 15 15. The process according to claim 14, wherein the gene(s)
is/are amplified by overexpression.
- 25 16. The process according to claim 11, wherein bacteria
are fermented for the production of L-lysine in which
one or more of the genes selected from the group
consisting of

a) the pck gene, which encodes phosphoenolpyruvate carboxykinase, and

b) the pgi gene, which encodes glucose 6-phosphate isomerase,

5 is/are simultaneously attenuated.

17. The process according to one of claims 8-12 or 14-15, wherein microorganisms of the genus *Corynebacterium glutamicum* are used.

10 18. A process for production of DNA of genes which encode phosphofructokinase comprising employment of polynucleotide sequences according to claim 1 as primers in a polymerase chain reaction.

19. A hybridization probe comprising a polynucleotide sequence according to claim 1.

15

Novel nucleotide sequences encoding the pfkA gene**Abstract**

An isolated polynucleotide containing a polynucleotide sequence selected from the following group:

- 5 a) a polynucleotide which is at least 70% identical to a polynucleotide which codes for a polypeptide containing the amino acid sequence of SEQ ID NO:2,
- b) a polynucleotide which encodes a polypeptide which contains an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID NO:2,
- 10 c) a polynucleotide which is complementary to the polynucleotides of a) or b), and
- d) a polynucleotide containing at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),
- 15

a process for the fermentative production of L-amino acids with amplification of the pfkA gene and use as primer or hybridisation probe.

Figure 1: Plasmid map of pEC-T18mob2

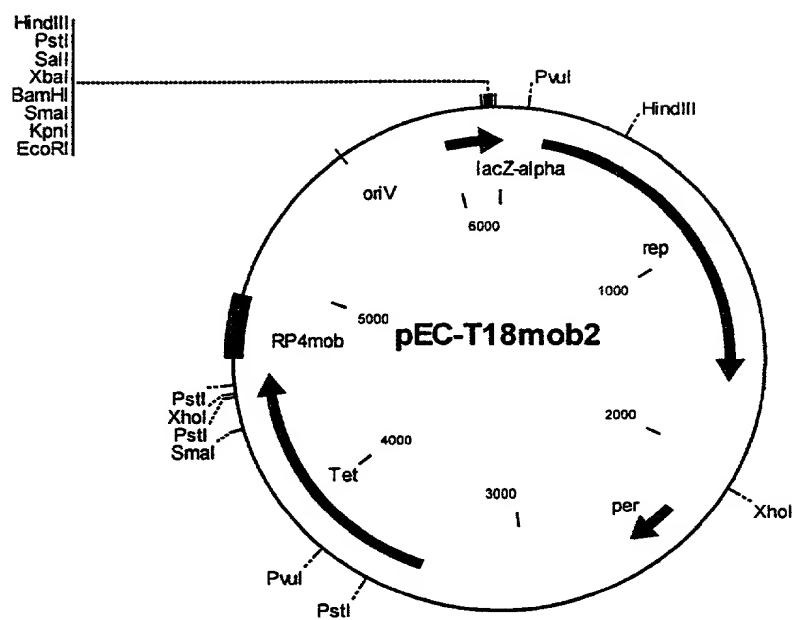


Figure 2: Plasmid map of pT-pfkAexp

